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AN EXAMINATION OF THE METHODS FOR THE MICROCHEMICAL DETECTION OF PHOSPHORUS COMPOUNDS OTHER THAN PHOSPHATES IN THE TISSUES OF ANIMALS AND PLANTS.

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The microchemical reaction for the detection of phosphorus in the tissues of animals and plants introduced in 1898 by Macallum ('98) is a modification of that devised in 1893 by Lilienfeld and Monti ('93). These investigators attempted to demonstrate the distribution of phosphorus in tissues by subjecting the latter for some time to the action of a solution of ammonium molybdate in nitric acid, after which they were treated with a solution of pyrogallic acid. The nitric molybdate reagent was supposed to liberate the phosphorus from its organic combinations, to convert it into orthophosphoric acid, and finally to precipitate the latter as the yellow phosphomolybdate of ammonium. The further treatment of the tissues with pyrogallic acid had for its object the reduction of the ammonium phosphomolybdate to a lower oxide of molybdenum, which, according to Lilienfeld and Monti had a brown or black color. In this way the pale yellow precipitate containing the phosphorus was converted into a dark-colored precipitate which could be easily studied under the microscope.

The importance of a microchemical reaction which would enable us to determine accurately the distribution of the compounds of phosphorus not only in the tissues but also in the parts of the cells of the tissues can hardly be overestimated. It is therefore

not surprising that the results of Lilienfeld and Monti have been subjected to careful experimental examination by a number of other investigators.

Raciborski ('93) in 1893, in his review of Lilienfeld and Monti's article, showed that the reaction of ammonium phosphomolybdate with pyrogallic acid resulted in the production of a green compound, while ammonium molybdate gave by reduction with the same reagent a brown compound. He concluded that the brown reaction of Lilienfeld and Monti was due to ammonium molybdate mechanically imbibed by the section, and not to ammonium phosphomolybdate.

Heine ('96), also, showed that phosphorus-free histone, prepared from the thymus, formed with the nitric-molybdate reagent compounds from which the ammonium molybdate could not be removed by washing in water, and in which it could be detected by the use of reducing compounds. For this purpose he employed stannous chloride.

Macallum confirmed Raciborski's observations as to the color compounds produced by the reduction of ammonium phosphomolybdate and ammonium molybdate respectively, and showed that ammonium molybdate could not be removed from tissues which had been treated with the nitric-molybdate reagent even by washing for several months in changes of distilled water. Macallum perceived the necessity of substituting for pyrogallic acid, which gives colored compounds with both the phosphomolybdate and the molybdate of ammonium, some reagent which would discriminate between these two compounds, and give a color reaction with phosphomolybdate alone. This condition he found to be fulfilled by zinc chloride, previously introduced for this purpose by Polacci ('94), which gave a green color with the phosphomolybdate but did not act on ammonium molybdate. Owing, however, to the fact that zinc chloride acted very slowly, he finally adopted as a reducing agent phenylhydrazin hydrochloride, which, according to him, made a very marked distinction, in the absence of alcohol or of caustic alkali, between the molybdate and the phosphomolybdate compounds. It gave with the former in powder the brown oxide at once, in solution, a brownish precipitate, which appeared at once, or later, according

to the strength of the solution. On a solution of the molybdate containing nitric acid, *e. g.*, that used as the reagent for phosphoric acid, it had no apparent effect on the molybdenum compound, although, in a few minutes, a soluble, reddish, aromatic compound might be formed in the solution. On the other hand, with phosphomolybdates, either in the presence or in the absence of ammonium molybdate, or of nitric acid, or of both, it gave at once the dark green oxide of molybdenum.

Concerning the use of these reagents on tissues Macallum says: "On the molybdate and phosphomolybdate compounds distributed in animal and vegetable tissues, the phenylhydrazin hydrochloride acts as it does on these in the test-tube. It is not necessary to free the tissue preparations from ammonium molybdate." He recommends washing the preparations for a minute or two in a dilute solution of nitric acid after which they are transferred to the reducing solution, which in less than two minutes, brings out the green color where the phosphomolybdate compound occurs, but a faint yellow reaction where ammonium molybdate alone is present.

The technique of the reaction is as follows: Fresh tissues or tissues hardened in alcohol were used. Pieces of tissue or thin sections in the case of hardened material, were placed, for a period varying from ten minutes to forty-eight hours, in a solution of ammonium molybdate in nitric acid, prepared by dissolving one part of pure molybdic acid in four parts of strong ammonia, and adding thereto, slowly, fifteen parts of nitric acid, sp. gr. 1.2. After the nitric molybdate reagent has acted for a sufficient length of time, the preparations are washed in water or in dilute nitric acid, and treated with a ¼ per cent. solution of phenylhydrazin hydrochloride, which reduces the phosphomolybdate to a green-colored oxide of molybdenum.[1] The tissues may be then dehydrated, cleared in oil of cedar and mounted in balsam.

According to Macallum, inorganic compounds of phosphorus

[1]Although Macallum speaks of a green oxide of molybdenum being formed by this reaction, it is probable that the bodies formed belong to the blue oxides. The green color obtained at the beginning of the reduction of phosphomolybdate of ammonium in vitro is due to the yellow background of unreduced molybdate, that obtained in the tissues to the associated xanthoproteic reaction (vide infra).

are first affected, then lecithins, and finally the organic compounds of phosphorus. Where it is desired to demonstrate the distribution of the latter he recommends the preliminary removal of the lecithins by repeated extraction with hot ethyl alcohol in a Soxhlet apparatus.

In the original form devised by Lilienfeld and Monti or in the form of Macallum's modification this reaction for phosphorus has been extensively employed. Macallum's original paper dealing with the reaction contains a considerable number of contributions dealing with the distribution of organic compounds of phosphorus in various tissues, and the reaction has been applied to the solution of special problems of this nature by Sherrington ('94), Gourlay ('94), Scott ('99), Held ('95), Bensley ('03), Wager ('05), Richter ('05), and many others. It is therefore of the utmost importance that every detail of the reaction should be carefully tested to exclude all possible sources of error.

Recently I have obtained results which have led me to suspect that the reaction obtained by Macallum's method is not wholly due to the formation in the tissue of ammonium phosphomolybdate, but that other compounds of molybdenum may be present which are capable of reduction to the blue oxide by means of phenylhydrazin hydrochloride. For example, I observed that the peripheral portions of sections gave uniformly a deeper and more diffuse reaction than the central portions. This result was first noted in the tips of the villi in sections of the small intestine, and was ascribed to the presence of inorganic phosphates absorbed from the food. Later it was noted that the same result was obtained in sections of the liver, pancreas, and other organs. Furthermore, I observed that freshly prepared solutions of the nitric molybdate often gave a strong reaction in the tissues after very short periods of immersion. For example, sections of the fundus region of the stomach of the rabbit, treated with warm, freshly prepared nitric molybdate reagent for ten minutes, then washed in water and reduced by means of a one per cent. solution of phenylhydrazin hydrochloride gave a diffuse bluish green reaction together with a strong reaction in the nuclei and in the granules of the parietal cells. This result was clearly not due to phosphorus compounds of any sort, because the same nitric

molybdate reagent after having been kept several days, during which it had deposited copious crusts of molybdic acid, gave, when applied to sections from the same source, no such result, the reaction proceeding in the slow progressive manner characteristic of the phosphorus reaction as described by Macallum. Again, in his investigation of the nature of the granule cells of Paneth, Mr. Klein, working under my direction, found it necessary to employ formalin in the fixation of the tissues in order to preserve the granules. In preparations of this material treated by Macallum's method, we were surprised to obtain a strong reaction in the fibrils of the collagenic tissue of the tela submucosa.

Clearly, the intense marginal reaction obtained in sections and the early reaction obtained when freshly prepared solutions of the nitric molybdic reagent were used could not be due to phosphorus. These anomalous characters of the reaction as applied to sections could only be explained on the assumption that, after the treatment with the nitric molybdic reagent, there existed in the tissue compounds of molybdenum other than phosphomolybdates, which gave the blue reaction with phenylhydrazin hydrochloride.

On account of the fact that the difference between freshly prepared and older solutions of the nitric molybdic reagent is to be found in the amount of molybdic acid contained, it seemed probable that the extraordinary results of the reaction, as described above, were due to the absorption of this substance by the tissue elements. Accordingly, I undertook experiments to determine the behavior of solutions of molybdic acid in reaction with phenylhydrazin hydrochloride, as well as the capacity of the tissue elements for absorbing it from its solutions. Later it was found necessary to reinvestigate the reaction obtained by treating ammonium molybdate in solution, and phosphomolybdate of ammonium suspended in water, respectively with solutions of phenylhydrazin hydrochloride.

Although the experiments were started with the expectation that a portion of the reaction would be found to be due to absorbed molybdic acid, I still thought at that time that the fundamental assumption was true, upon which the reaction was based, namely, that the organic phosphorus was liberated from its com-

binations by the reagent, converted into orthophosphoric acid and immediately precipitated *in situ* as ammonium phosphomolybdate. As a result of the experiments, however, I have been forced to the conclusion that the whole of the reaction obtained by Macallum's method is due to compounds of molybdenum other than phosphomolybdate and that the phosphorus of the tissues is not concerned in the production of the reaction at all.

For the purpose of testing the reactions of molybdic acid with phenylhydrazin hydrochloride, I prepared two soluble molybdic acids. The first of these was prepared by the method recommended by Ullik ('67). Barium molybdate, prepared by precipitating a warm solution of ammonium molybdate with barium chloride washing thoroughly with hot distilled water, and drying the precipitate on a water bath at 100° C., was suspended in water and decomposed with its equivalent of sulphuric acid. The solution was then filtered, tested for barium, sulphuric acid and chlorides, from which it was found to be free, and the total acidity was determined by titration with a normal solution of sodium hydroxide, using phenolphthalein as indicator. Assuming that the solutions contained a molybdic acid having the formula $H_2Mo_2O_7$ the concentration of the solutions obtained by the method described above was in the case of one preparation 5.25 per cent., in another 7.52 per cent. The second molybdic acid was colloidal molybdic acid prepared by the process recommended by Graham (64), except that ammonium molybdate was employed instead of sodium molybdate. A solution of ammonium molybdate in hydrochloric acid was dialyzed for several days against distilled water, until free from chloride. The resulting solution was then titrated against normal soda solution using phenolphthalein as indicator. According to Sabanejew ('90) the molecular weight of the molybdic acid prepared by Graham's method as determined by lowering of freezing point is 620 corresponding to the formula $H_2Mo_4O_{13}$. Assuming that the same compound was obtained by the dialysis of the solution of ammonium molybdate in hydrochloric acid the solution obtained contained 6.73 per cent. of colloidal molybdic acid. From these solutions were prepared the various solutions mentioned in the succeeding experiments.

Solutions of both molybdic acids give, when treated with phenylhydrazin hydrochloride an immediate blue reaction which gradually deepens in color and a blue precipitate forms.

Sections of tissues fixed in alcohol, cut in paraffin, and fastened to the slide by the water method were placed in each of the solutions of molybdic acid. From time to time sections were removed from the solution, rinsed in water, and tested with a 1 per cent. solution of phenylhydrazin hydrochloride. It was found that the molybdic acid was taken up by the tissues from both solutions and was detectable in them by the blue reaction obtained by reduction with phenylhydrazin hydrochloride. In sections treated with pure solutions of soluble or of colloidal molybdic acid the strongest reaction was obtained in the collagenic fibrils which were deep blue. A slight diffuse reaction was obtained in the cytoplasm of cells, and a somewhat stronger reaction in the nuclear chromatin. The amount, however, of molybdic acid taken up from dilute pure solutions was not great except as regards the collagenic tissue. The experiments show, however, that molybdic acid may be taken up from its solutions by tissues and may be detected in these by the blue reaction produced by treatment of the sections with phenylhydrazin hydrochloride.

Under the conditions of the Lilienfeld-Monti-Macallum reaction molybdic acid occurs in the solution associated with nitric acid as well as with ammonium molybdate, ammonium nitrate, and the products of dissociation of all these compounds. Accordingly, the effect of the presence of acids on the absorption of the molybdic acid from its solutions by tissues was tested. Sections were placed in solutions of soluble and of colloidal molybdic acid to which five per cent. of nitric or of hydrochloric acid had been added, and were tested from time to time with phenylhydrazin hydrochloride. I found that the addition of either nitric acid or hydrochloric acid to the solutions of molybdic acid produced a remarkable increase in the capacity of sections for combining molybdic acid, which was again detectable by the blue or green reaction obtained by reduction with phenylhydrazin hydrochloride. With the mixture of nitric and molybdic acids the reaction obtained after reduction was a deep greenish blue color in the nuclear chromatin, a faint greenish blue in the cytoplasm,

and a deep blue in the collagenic fibers. Except for the strong reaction in the connective tissue, the result obtained by treatment of sections with a solution of molybdic acid containing nitric acid, followed by reduction in one per cent. phenylhydrazin hydrochloride was exactly similar to the so-called phosphorus reaction obtained by the procedure recommended by Macallum. It may be noted that the color obtained by the use of molybdic acid containing nitric acid followed by reduction differed from that produced by reduction of molybdic acid by phenylhydrazin in the test tube, inasmuch as the former gives a greenish blue color, the latter a pure blue. This difference was obviously due to the yellow background afforded by the xanthoproteic reaction. As in the phosphorus reaction, the absorption of the molybdic acid was progressive, the reaction after eighteen hours being much stronger than after three hours.

Similar results were obtained with solutions of molybdic acid containing hydrochloric acid, except that the molybdic acid was taken up much more rapidly from the hydrochloric solution than from the nitric solution, and that the resulting reaction was blue rather than greenish blue, owing to the absence of the yellow zanthoproteic reaction. A further difference exhibited itself in the fact that sections left for some time in the solutions developed the blue color *without the use of any reducing agent*, the organic compounds of the tissue evidently acting as reducers. In the presence of nitric acid this, of course, could not occur because of the strong oxidative action of this compound.

Thus, in sections treated with solutions of molybdic acid containing either hydrochloric, or nitric acid, followed by phenylhydrazin hydrochloride, results were obtained which were the exact counterpart of the results of the so-called phosphorus reaction, although there could be little possibility of the formation of precipitates of ammonium phosphomolybdate in the tissues. Curiously enough, the anomalous characters occasionally observed in the reaction obtained by Macallum's method were to be found in the molybdic acid material, that is to say, the more intense diffuse reaction of the outer portions of the sections and the deep reaction in the connective tissue. It seemed clear from these experiments that a portion, at least, of the result

obtained by the procedure of Macallum was due to absorption of molybdic acid from the nitric molybdate solution. There was, however, some possibility that even this reaction with molybdic acid solutions depended on the presence of phosphorus and its liberation as phosphoric acid. It might be supposed that this phosphoric acid reacted with the molybdic acid to produce phosphomolybdic acid which was in turn precipitated by the albumens of the tissues. Or it might even be supposed that ammonium phosphomolybdate was formed, the ammonium ions necessary to the reaction being furnished by the albumens. That this is not the case, and that the reaction obtained by the use of molybdic acid solutions is in no way dependent on the phosphorus content of the tissue, I think the following experiments will show.

In studying the reaction of solutions of molybdic acid with phenylhydrazin hydrochloride, I found that the addition of nitric acid to the mixture retarded the reaction and if a sufficient quantity were present prevented it altogether. Accordingly, experiments were undertaken to determine the limits of this reaction with molybdic acid, ammonium molybdate, and ammonium phosphomolybdate, respectively, in the hope that a sufficient difference in the behavior of these compounds would be discovered to enable one to employ a solution of phenylhydrazin hydrochloride containing enough nitric acid to inhibit the reduction of molybdic acid and of ammonium molybdate while permitting the reduction of ammonium phosphomolybdate, and thus discriminate between these compounds occurring in tissues treated by Macallum's methods.

I found that with a constant concentration of phenylhydrazin hydrochloride, the amount of nitric acid required to prevent the reduction to the blue oxide of molybdenum varied directly with the concentration of the molybdic acid but was constant for any given concentration.

I found, moreover, that the blue reduction was invariably obtained in solutions of ammonium molybdate containing nitric acid, when they were treated with solutions of phenylhydrazin hydrochloride, provided the amount of nitric acid did not exceed a certain amount, which, as in the case of molybdic acid, was

constant for a given concentration of the other two constituents but varied directly with the concentration. This fact made it necessary to determine the conditions of this reaction with ammonium molybdate if solutions of the hydrochloride containing nitric acid were to be used for the reduction of the sections, because in trying to eliminate the reduction of the molybdic acid by using a solution of the hydrochloride containing nitric acid, a new source of error might be introduced, inasmuch as the nitric acid would make ammonium molybdate available to the blue reduction.

The experiments to determine the limits of these reactions were made in test-tubes in much the same way as experiments in hæmolysis are carried out. In each of a series of test-tubes was placed, from a pipette graduated in fiftieths of a cubic centimeter, a measured quantity of the solution of molybdic acid. To this was added a quantity of nitric acid solution of known strength increasing by increments of one tenth of a cubic centimeter from tube to tube. Sufficient distilled water was then added to make the contents of each tube up to 9.5 c.c., and finally 0.5 c.c. of a two per cent. solution of phenylhydrazin hydrochloride was added to each tube. In such a series of tubes, if a sufficient range of concentrations of the nitric acid was included the tubes at one end of the series would give the blue reduction, those at the other would at first show no signs of reaction, but after several hours would develop a brownish color. At some point in the series two tubes, side by side, differing from one another only in the nitric acid content, would present the one a blue, the other a brown color. When solutions of nitric acid containing 32 grammes of nitric acid per 100 c.c. were employed, that is, when the difference in the contents of two tubes amounted to .032 gr. of nitric acid, the contrast in color between the tubes which marked the limit of the reaction was a very striking one. Attempts to define this limit more accurately by the use of more dilute solutions of nitric acid, and accordingly smaller increments, of nitric acid from tube to tube did not result more satisfactorily. For example in a series of tubes in which the increment of nitric acid was 0.012 gr. from tube to tube, the transition was distributed over several tubes, those immediately preceding the first

tube free from the blue precipitate containing a slight blue precipitate which subsided after several hours leaving a brownish supernatant fluid. Thus, the possible error in determining the proportion of nitric acid necessary to prevent the blue reaction is considerable, although the results are accurate enough for the purposes of this investigation, as subsequent statements will show.

Reactions of Soluble Molybdic Acid with Phenylhydrazin Hydrochloride in the Presence of Nitric Acid.

Strength of Molybdic Acid in Fractions of Normal.	Percentage of Phenylhydrazin Hydrochloride.	Percentage of Nitric Acid Necessary to Prevent Blue Reaction.
0.02	0.1	1.38
0.04	0.1	2.26
0.06	0.1	2.94
0.08	0.1	3.27

Reactions of Solutions of Ammonium Molybdate with Phenylhydrazin Hydrochloride in the Presence of Nitric Acid.

Solutions of ammonium molybdate when treated with phenylhydrazin hydrochloride give, as stated by Macallum, a brown color, and a brown precipitate slowly forms in the solution. In the presence of nitric acid, however, provided the latter does not exceed a certain amount which varies with the concentration of the molybdate, the reaction consists in the production first of a blue color and finally of a deep blue precipitate. If the amount of nitric acid exceeds this maximum no reaction occurs at first, but a brown color slowly develops in the solution. The following table gives the concentration of nitric acid necessary to prevent wholly the blue reaction with several concentrations of molybdate.

Percentage of Ammonium Molybdate.	Percentage of Phenylhydrazin Hydrochloride.	Per Cent. Nitric Necessary to Prevent Blue Reaction.
0.5	0.1	3.14
1.0	0.1	4.40
1.5	0.1	5.03
2.0	0.1	5.66

Reactions of Phosphomolybdic Acid and Phosphomolybdate of Ammonium with Phenylhydrazin Hydrochloride.

Phenylhydrazin when added to a solution of phosphomolybdic acid or to crystals of ammonium phosphomolybdate suspended in water gives at once the reduction to the blue oxide. The reaction under these conditions proceeds so rapidly that it is difficult to follow the steps. The resulting body is not green in color as described by Macallum but blue, the green color which is first seen when phenylhydrazin hydrochloride is added to the crystals of ammonium phosphomolybdate being simply due to the yellow background afforded by the unreduced phosphomolybdate. After a few minutes the reaction proceeds still further and a soluble blue-violet compound is formed.

In the presence of nitric acid, the reaction proceeds somewhat more slowly, although it is less sensitive to the presence of nitric acid than the corresponding reactions with molybdic acid and ammonium molybdate. With crystals of phosphomolybdate suspended in water and 0.1 per cent. of phenylhydrazin the concentration of nitric acid may be increased to 36 per cent. without the reduction to the blue oxide being prevented. Even in the presence of this great amount of nitric acid the reduction of the phosphomolybdate reaches a maximum within ten minutes after the phenylhydrazin hydrochloride is added. The reaction between phenylhydrazin and phosphomolybdic acid proceeds in much the same way, and is similarly much less sensitive to the presence of nitric acid, than the corresponding reactions with molybdic acid and ammonium molybdate. For purposes of comparison the experiments were made the results of which are presented in the subjoined table.

Percentage of Phosphomolybdic Acid.	Percentage of Phenylhydrazin Hydrochloride.	Per Cent. Nitric Acid Necessary to Prevent Blue Reaction.
0.1	0.1	6.55
0.2	0.1	9.82
0.3	0.1	13.10
0.4	0.1	14.74

On comparison of this table with the preceding one it will be seen that the reduction of phosphomolybdic acid having a con-

centration of 0.1 per cent. will proceed in the presence of nitric acid having a concentration sufficient to prevent reduction in a solution of ammonium molybdate of 2 per cent. strength.

While it would have been difficult to draw from these experiments conclusions as to the probable behavior of the compounds of molybdic acid in the tissues when treated with solutions of phenylhydrazin hydrochloride containing nitric acid, yet they suggested a possibility which was capable of being proved experimentally that the compounds of molybdic acid and molybdates found in the tissues would fail to react to phenylhydrazin hydrochloride in the presence of an amount of nitric acid which would have no effect on the reduction of ammonium phosphomolybdate.

In order to test this question, sections of the liver of Necturus prepared after fixation in alcohol and fastened to the slide by the water method were treated with Macallum's nitric molybdate reagent, a solution of soluble molybdic acid in 10 per cent. nitric acid, and a ten per cent. solution of phosphoric acid, respectively. The two first mentioned solutions were allowed to act for three hours at 37.5° C. followed by eighteen hours at ordinary room temperature. They were then tested with a 0.1 per cent. solution of phenylhydrazin hydrochloride and found in each case to give a strong reaction corresponding in its characters and distribution to the phosphorus reaction of Macallum. The reaction obtained in the sections treated with the solution of molybdic acid was much the stronger. Other sections from the same lot were then treated with solutions containing 0.1 per cent. of phenylhydrazin hydrochloride and varying known quantities of nitric acid, in each case for a period of fifteen minutes. The sections from the molybdic acid solution and from the nitric molybdate reagent were treated side by side in the same solution, and for purposes of control a section which had been soaked in phosphoric acid and then treated with the nitric molybdate reagent was also put at the same time in the solution, so that it was possible to observe the effect of different concentrations of nitric acid on the reduction of sections treated with molybdic acid, or with the nitric molybdate reagent, and of sections containing ammonium phosphomolybdate artificially introduced. It was my expectation that a low concentration of nitric acid would suffice to abolish that portion of the reac-

tion which was due to ammonium molybdate and to molybdic acid, and that a considerable residuum of the reaction would be found unaffected by even high concentrations of nitric acid and could thus be interpreted as a true phosphorus reaction. I was quite unprepared for, and greatly disappointed at the actual result of these experiments, namely, that relatively low concentrations of nitric acid abolished the reaction altogether.

With a concentration of 3.27 per cent. of nitric acid, phenylhydrazin hydrochloride 0.1 per cent., the molybdic acid sections and the nitric molybdate section showed no reaction after three minutes' treatment, although the section containing ammonium phosphomolybdate artificially introduced gave a maximum reaction in less than one minute. After fifteen minutes' action, a very faint reaction was obtained in the nuclei, both in the molybdic acid section and in the nitric molybdate section. When the concentration of the nitric acid reached 16.37 per cent., the phenylhydrazin remaining the same, the reaction was not recognizable after fifteen minutes' treatment although sections containing ammonium phosphomolybdate artificially introduced reduced to a maximum depth of color in the same solution in five minutes. I have repeated these experiments many times, always with the same results. It is significant that the reaction disappeared at exactly the same point as regards concentration of nitric acid in the molybdic acid section and the nitric molybdate section.

Only one conclusion is possible from these experiments, namely, that sections after treatment with Macallum's reagent for this length of time did not contain appreciable quantities of ammonium phosphomolybdate. Thus the fundamental assumption on which the reaction of Lilienfeld and Monti and of Macallum is based falls to the ground. It is obvious that if the phosphorus of the organic compounds is liberated at a point short of the destruction of the recognizable structures of the cell, it is not, at all events, precipitated *in situ* by the nitric molybdate reagent.

As a result of these experiments I am of the opinion that the reaction obtained by Macallum's procedure is entirely due to the formation of compounds of molybdic acid with the albumens of the tissue and not in any respect to the formation of ammonium phosphomolybdate at the expense of the organic phosphorus.

The facts on which the conclusion is based are, briefly, as follows :

The essential conditions of a successful phosphorus reaction are, first, that the phosphorus may be liberated from its organic combinations at a point short of the destruction of the recognizable structure of the cell ; second, that the liberated phosphorus be precipitated at once at the point of origin as ammonium phosphomolybdate ; third, that the reducing substance employed to make the phosphomolybdate visible for microscopic study act on phosphomolybdate and on no other compound of molybdenum which may be present in the tissue.

Phenylhydrazin hydrochloride does not meet the third condition because it reduces to the blue oxide of molybdenum, soluble molybdic acid in the test tube as well as molybdic acid combined with the tissue constituents in sections.

Phenylhydrazin hydrochloride also produces the blue oxide when treated with ammonium molybdate in the presence of nitric acid, provided that the latter does not exceed a certain concentration which is constant for constant concentrations of the molybdate.

Nitric acid affects the reduction of molybdic acid, ammonium molybdate, and ammonium phosphomolybdate, by phenylhydrazin hydrochloride in the same way, namely, retards the reduction, but to different degrees, inasmuch as low concentrations of nitric acid *prevent* the reduction of the two former to the blue oxide, while high concentrations of nitric *merely retard* the blue reduction of the phosphomolybdate. Accordingly, if phosphomolybdate is formed at the site where a reaction is obtained by the method of Macallum, the reaction ought to be elicited by treatment of the sections with solutions of phenylhydrazin in having a high content of nitric acid. This, however, the experiments show is not the case. Even low concentrations of nitric acid eliminate the greater portion of the reaction, and the reaction is entirely abolished by a nitric acid content which has little effect on the reduction of phosphomolybdate of ammonium artificially introduced into sections for purposes of control. Furthermore, the reaction is abolished at the same concentration of nitric acid with sections treated with Macallum's nitric molybdate reagent

as with sections treated with a pure solution of molybdic acid. These facts dispose finally of the first and second essential conditions of a successful microchemical reaction for organic phosphorus, for it is clear that if the sections after treatment with the reagent contain no phosphomolybdate of ammonium, that the organic phosphorus has either not been liberated from its compounds, or that, if it has, it has not been precipitated at the moment and at the point of liberation. If these conclusions are correct, it is also obvious that there is no hope of a real phosphorus microchemical reaction being obtained by the employment of the nitric molybdate reagent.

These conclusions do not, of course, apply to the identification of phosphates by the nitric molybdate reagent, in cases where the characteristic crystal form of the ammonium phosphomolybdate can be recognized under the microscope.

In conclusion, it may be mentioned that in making the experiments to determine the effect of nitric acid on the reduction of the molybdenum compounds by phenylhydrazin hydrochloride it is important to employ solutions which are free from nitrous acid, which reacts with the phenylhydrazin and reduces its concentration.

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